QUANTITATIVE ANALYSIS OF TRANSLATIONAL COUPLING IN *ESCHERICHIA COLI*

BY

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THESIS

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Abstract

Multiple genes are often transcribed on the same mRNA transcript in bacteria. These multi-gene transcriptional units, called operons, are widespread in bacteria with nearly half of all annotated genes predicted to reside in them. How operons affect translational regulation is still not known. There is a phenomenon specific to operon, so called translational coupling, which means that the translation of the downstream gene is often dependent upon the translation of the upstream gene. As a step towards developing a model for protein expression pattern in operon, we have engineered a synthetic two-gene operon, where translation of the downstream gene is conditional on the translation of the upstream gene. This operon encodes two fluorescent proteins, where the upstream gene is translated by orthogonal ribosomes and the downstream gene by native ribosomes. This design allows us to precisely tune the translation of the upstream gene and then record its affect on the translation of the downstream gene. Using this general system, we show that the translation of these two genes can be tightly coupled. We have also showed that the mRNA level is also tightly coupled with the gene expression level, which indicates the correlation between the amount of ribosome translating and the mRNA stability.

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This work can never be done without the help of many people. I would like to first thank my advisor, Christopher Rao, for giving me the initial idea of this work and patiently training and advising me along my way struggling for this project. I have leaned so many things from you, such as the way engineers think of biology systems, how to find new ideas from existing knowledge pool, how to deal with failure and stress, how to present work to different audience and more. I would also like to thank Khushnuma Koita for the beginning lab work training and all the days we spent together chating in the lab and sweating in the gym. Now you are going to graduate, wish you have a happy life and bright future and be always so cool. I would also like to thank Lon Chubiz, for educating me research expertise. Special thank to your work of developing the orthogonal ribosomes. My project is the step followed by that work. Thank Kang Wu for many valuable suggestions and critical opinions. Thank Supreet Saini for helping me trouble shooting many experiment failures and helps with the teaching assistantship. Thank Yuki Kimura for teaching me programming and building the lab website, server, backup systems and all the technical support. We all benefit from your beautiful work. Thank Ahmet Badur, Kori Dunn and Santosh Koirala for helping me with experiments and advices.

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Table of Contents

Chapter 1: Introduction

1.1 Project Motivation

Protein translation is always a fundamental topic in the biology kingdom. As engineers, we want to use bacterial as tools to synthesize value added products, such as proteins or small chemicals that can be used as pharmaceuticals, transplant materials, biofuels and etc. All of the applications involve protein synthesis, no matter they are the end products or the enzymes that make the products. Translation is the process which ribosomes synthesize peptides from the template mRNA. It is the important final step in protein synthesis. Yet there still remain many mysteries in its mechanism.

The existing models for predicting protein expression level mainly account for the sequence in translation initiation region (TIR) on a given mRNA, mRNA secondary structure, codon bias, riboswitches, small noncoding RNAs and etc [1-5]. We hypothesize that any model cannot ignore the operon structure. Operon is the structure that multiple genes are transcribed on the same mRNA. It is unique in prokaryotes, while in eukaryotes one gene is usually transcribed on one mRNA. *Escherichia coli* genome encodes about 4000 genes [6] and it is predicted that there are 630-700 operons [7]. Therefore towards developing such a new model for protein expression, we need to analyze the operon regulation.

There is a unique phenomenon in operon, the translational coupling [8]. It means that the translation of adjacent genes can affect each other. We want to determine the degree of coupling between adjacent genes on the same mRNA. We also want to explore why they are coupled. These knowledge will greatly help us understand protein synthesis and will serve as the theory

for developing models predicting protein expression in *Escherichia coli.* It will finally benefit synthetic biology and the industry that utilizes microorganisms as tiny machinery to synthesize specific molecules.

1.2 Background

Protein synthesis can be divided into two steps basically: transcription and translation. Transcription is the process that RNA polymerase replicates messenger RNA (mRNA) from the template DNA. Translation is the process that ribosome incorporates individual amino acids into a peptide according to mRNA template. The former step has been characterized extensively by previous researchers. A number of computational and experimental tools have been developed, including motif finding algorithms, reporter genes, microarrays and gel-shift assays [9, 10]. In the area of synthetic biology, promoter engineering has been widely used to achieve controlled protein expression level [11, 12].

However less is known about translation regulation. In the area of translation regulation, there is special phenomenon in prokaryotes, translational coupling [8, 13]. In bacterial multiple genes are often transcribed on one mRNA. These multi-gene transcriptional units are called operon. In an operon, translation of adjacent genes can affect each other. This is called translational coupling. For example mutations of the upstream gene can decrease the translation of the downstream gene in *Escherichia coli*, and vice versa [8].

In order to study translational coupling in *Escherichia coli*, we need tools to tune the translation strength of a given gene on an operon. The most straight forward way is engineering different translation initiation region (TIR). In *Escherichia coli*, the most recognized motif for translation initiation is the Shine-Delgarno (SD) sequence [14]. It is complementary to the 3’ end of the 16S

rRNA in the small subunit of the ribosome [15, 16]. On the rRNA, the sequence is CCUCCU, therefore SD sequence mostly resembles its complementary sequence AGGAGG. Based on this knowledge, we can selectively engineer different ribosome binding site (RBS) and tune the translation of the gene we want [17]. However it should be pointed out that many genes lacking the recognized SD sequence still translate efficiently [18-21]. Therefore any translation initiation regions used in this study need to be characterized individually. Again this is also what we want to address: how the operon regulation can solve the mystery partially.

Another powerful tool to tune the translation of a certain gene is the orthogonal ribosomes [22-26]. It is able to translate specific genes that the host ribosome cannot recognize. In this project, we used the orthogonal ribosome that was previous developed in this lab [26]. It was computationally designed based on the binding strength between the 3' end of the 16S rRNA and the ribosome binding site (RBS) from the mRNA. There are three rules in the design: i) the binding strength of the orthogonal ribosome-RBS of mRNA is in the similar magnitude of the host ribosome-RBS of the mRNA; ii) The orthogonal ribosome does not bind to mRNA on the host Shine-Delgarno sequence; iii) The orthogonal ribosome minimally interacts with the rest of the translation initiation region on the host mRNA. The orthogonal ribosome is expressed as a mutated version of the rrnB operon and driven by an inducible promoter [26]. It enables us to control the expression of the orthogonal ribosome, further control the expression of the gene with orthogonal ribosome binding site.

With these tools in hand, we want to explore translational coupling by placing two fluorescent reporter genes on the same mRNA and look for the degree they are coupled in *Escherichia coli*. One of the genes can be controlled by different ribosome binding site or orthogonal ribosomes and both protein expressions can be monitored by fluorescence measurements quantitatively.

Furthermore we want to explore the mechanism why the translation of the two genes can be coupled.

Chapter 2: Material and Methods

2.1 Bacterial Strains, Media and Growth Conditions

All cloning steps were performed in *E.coli* LC100 (F- *ilvG rfb-50 rph-1 attB$_\lambda$*::[P_{N25}-*tetR lacIq spcR*]) [26]. LC100 was constructed previously in our lab by P1*vir* transduction of the chromosomally integrated TetR/LacI expression cassette from DH5αZ1 into strain MG1655 [6]. Cultures were grown in Luria-Bertani (LB) liquid media (composition: 10.0 g tryptone, 5.0 g yeast extract, 10.0 g sodium chloride in 1L solution). Media were supplemented with 25 µg/ml chloramphenicol, 100 µg/ml ampicillin for selection in *E. coli* when required. Inducers anhydrotetracycline (aTc) and isopropyl-β-D-galactopyranoside (IPTG) were used at concentration of 100 ng/mL and 1mM, respectively, unless otherwise specified. All cultures were grown at 37°C.

2.2 Cloning Procedure

All primers were purchased form Integrated DNA Technologies. All restriction enzymes were purchased from New England BioLabs, and all digestions were performed according to standard protocols. PCRs were performed by Phusion high-fidelity DNA polymerase (New England BioLabs). Ligations were performed overnight in 16°C by T4 DNA ligase (Fermentas). Gel extractions were performed by Zymoclean Gel DNA recovery kits (Zymo Research Corporation). Minipreps were performed by GeneJET Plasmid Miniprep Kit (Fermentas). Sequencings were performed by ACGT Inc.

2.3 Plasmid Construction

Reporter protein venus [27] was amplified using primers SZ006F/SZ009R, bearing EcoRI and HindIII restriction sites. The PCR fragment was cloned into pPROTet.E (Clontech), resulting the plasmid pPROTet-S-venus. The plasmids pPROTet-M-venus and pPROTet-W-venus were constructed in the same way by primer SZ007F/SZ009R, SZ008F/SZ009R respectively.

In the plasmid pPROTet-S-venus-S-mCherry, the reporter protein mCherry was a modified version from the original mCherry [28]. The 18^{th} and 21^{st} nucleotides were mutated into A and A respectively in order to get rid of the internal translation start site without changing the amino acids. This version of mCherry was amplified by primer SZ257F/SZ260R. The pPROTet-S-venus plasmid was amplified by primer SZ259F/SZ256R. The resulting two PCR fragments were assembled together by Gibson's one step enzymatic assembly methods [29], creating an additional stop codon just behind venus gene and a 44 bp spacer between venus and the strong ribosome binding site of mCherry gene. pPROTet-S-venus-W-mCherry was constructed similarly except that mCherry was amplified by primer SZ258F/SZ260R. pPROTet-W-venus-S-mCherry and pPROTet-W-venus-W-mCherry were constructed similarly with the above description except that the PCR template for vector is pPROTet-W-venus.

To construct the plasmid pZE-O-venus-S-mCherry, three DNA fragments were ligated. pZE12 plasmid [30] was digested by EcoRI and NotI. The O-venus was amplified by primer SZ015F/SZ112R, bearing EcoRI and HindIII restriction sites. The S-mCherry was amplified by primer SZ098F/SZ014R, bearing HindIII and NotI restriction sites. A three way ligation was performed and yielded pZE-O-venus-S-mCherry, with the intergenic distance 55 bp.

2.4 Fluorescence and Cellular Growth Measurements

To measure fluorescent protein expression, cultures were grown overnight in noninducing LB media. Cultures were diluted 1:100 (final) in fresh LB without inducers in the shaking 96 deep well plate in 37°C for 1.5 hours. Then equal volumes of fresh LB media with inducers were added and the cultures were then allowed to shake continuously for 3 hours before fluorescence and optical density measurements. All measurements were performed by Tecan Safire2 plate reader.

For venus fluorescence, the excitation wavelength was 515 nm and emission wavelength was 528 nm. The gain was set to 100. For mCherry fluorescence, the excitation wavelength was 587 nm and emission wavelength was 610 nm. The gain was set to 180. All measurements were recorded from an average of 9 reads and all cultures were done in quadruplicates.

2.5 The mRNA Quantification (qRT-PCR)

From each sample, 200 µl cells were taken directly from the cultures from fluorescence measurements, and total RNA was extracted by RNeasy Protect Bacteria Mini Kit (Qiagen). The resulting RNA was treated by TURBO DNA-free kit (Ambion) to clear the genomic and plasmid DNA. Eight hundred nanograms of RNA from each sample was used for a reverse transcription with QuantiTect Reverse Transcription Kit (Qiagen), with venus specific primer SZ070R or mCherry specific primer SZ071R. A 1µl sample from each reaction mixture was used to set up a quatitative PCR with HotStart-IT SYBR Green qPCR Master Mix with UDG (2X) (Affymetrix), with no reverse transcriptase control. The primers for venus amplicon are VF5/VR5, and mCherry MF1/MR1. The reactions were run with MJ Mini Opticon (Bio-rad) in triplicates. Data were analyzed with Opticon Monitor 3 (Bio-rad).

2.6 Tables

Table 1. Strains used in this study.

Strain	Genotype or plasmids contained	Source or reference
LC100	F- *ilvG rfb-50 rph-1 attB$_{\lambda}$*::[P_{N25}-*tetR lacIq spcR*]	Chubiz
SZ001	LC100/pPROTet-S-venus	This study
SZ002	LC100/pPROTet-M-venus	This study
SZ003	LC100/pPROTet-W-venus	This study
SZ004	LC100/pPROTet-S-venus-S-mCherry	This study
SZ005	LC100/pPROTet-S-venus-W-mCherry	This study
SZ006	LC100/pPROTet-W-venus-S-mCherry	This study
SZ007	LC100/pPROTet-W-venus-W-mCherry	This study
SZ008	LC100/pZE-O-venus-S-mCherry and pOR1	This study

Table 2. Plasmids used in this study.

Plasmid	Relevant characteristics	Source
pPROTet.E	*cm* $P_{LtetO-1}$ ori ColE1	Clontech
pPROTet-S-venus	pPROTet.E derivative, strong RBS -venus	This study
pPROTet-M-venus	pPROTet.E derivative, medium RBS - venus	This study
pPROTet-W-venus	pPROTet.E derivative, weak RBS - venus	This study
pPROTet-S-venus-S-mCherry	pPROTet.E derivative, strong RBS - venus - strong RBS - mCherry	This study
pPROTet-S-venus-W-mCherry	pPROTet.E derivative, strong RBS - venus - weak RBS - mCherry	This study
pPROTet-W-venus-S-mCherry	pPROTet.E derivative, weak RBS - venus - strong RBS - mCherry	This study
pPROTet-W-venus-W-mCherry	pPROTet.E derivative, weak RBS - venus - weak RBS - mCherry	This study
pZE12	*bla* $P_{LlacO-1}$ ori ColE1	[30]
pZE-O-venus-S-MCherry	pZE12 derivative, orthogonal RBS - venus - strong RBS - mCherry	This study
pOR1	*cm* $P_{LtetO-1}$ ori p15A orthogonal ribosome	[26]

Table 3. Primers used in this study.

Primer	Sequence
SZ006F	agctgaattctaaaaggaggagaaaatgagtaaaggagaagaact
SZ007F	agctgaattccaaattaaatattttatgagtaaaggagaagaact
SZ008F	agctgaattccaaagccgatcccccatgagtaaaggagaagaact
SZ009R	atccaagcttttatttgtatagttcatcca
SZ014R	tatgcggccgcttacttgtacagctcgtcca
SZ015F	actagaattctaagtctcgaaaaaaatgagtaaaggagaagaact
SZ070R	ttatttgtatagttcatccat
SZ071R	ttacttgtacagctcgtccatgc
SZ098F	atccaagctttaaaaggaggagaaaatggtgagcaagggcgaaga
SZ112R	atccaagcttcgcagattgtttctggggctattatttgtatagttcatcca
SZ256R	aagcttagcatcaggatcccgcagattgtttctggggctattatttgtatagttcatcca
SZ257F	tgcgggatcctgatgctaagctttaaaaggaggagaaaatggtgagcaagggcgaagaag
SZ258F	tgcgggatcctgatgctaagcttcaaagccgatcccccatggtgagcaagggcgaagaag
SZ259F	gcggccgcttaattaattaatc
SZ260R	tgcctctagattaattaattaagcggccgcttacttgtacagctcgtcca
VF5	tgatgcaacatacggaaaac
VR5	tggcactcttgaaaaagtca
MF1	agatcaagcagaggctgaag
MR1	tgttccacgatggtgtagtc

Chapter 3: Results

3.1 Tune Translation by Initiation Site

Towards studying translation coupling, the first step is to find a tool to control the expression of certain genes at translational level. The first tool we chose is engineering protein translation initiation region. The translation initiates when the 16S ribosome small subunit binds to a specific region, which is referred to ribosome binding site (RBS). Three RBS were picked, strong RBS "AGGAGGAGAAA", medium RBS "TTAAATATTTTA" and weak RBS "GCCGATCCCCCA" [31]. The strong RBS comes from our lab experience, and the medium and weak RBS come from Barrick's paper [31], from which they studied the strength of different RBS in *E. coli*. Medium RBS is No. 1243, and weak RBS is No. 1413 according to Barrick. These RBS were engineered in front of fluorescent protein venus, under the aTc inducible promoter $P_{LtetO-1}$, resulting pPROTet-S-venus, pPROTet-M-venus and pPROTet-W-venus, respectively. The fluorescence and optical density of the three strains were measured (Table 4). The venus fluorescence was normalize to optical density OD600. Indeed the strong RBS resulted in highest expression. The medium RBS drove venus expression about twice as much as the weak RBS strain. This experiment confirmed the first tool to tune the translation of a gene by varying ribosome binding site without changing other regulators. In the following studies, the strong and weak RBS were chosen to be the two representatives.

3.2 Characterization of Two-gene Operon Expression

To study how the translation of two genes on one mRNA can influence each other, a set of two-gene operons was constructed. As shown in Fig 1a, the two fluorescent protein venus and mCherry were constructed tandemly under the $P_{LtetO-1}$ promoter. Each protein was driven by

either a strong or a weak RBS, therefore resulting in four constructs. Their fluorescence were measured, and venus and mCherry expression were all normalized to the S-venus-S-mCherry construct (Fig. 1b). Comparing S-venus-S-mCherry and W-venus-S-mCherry, the latter one's venus was indeed suppressed greatly because the RBS for venus was weak. The interested point is that mCherry was also low (about 21 % of the S-venus-S-mCherry), while its own RBS remained the same. Comparing S-venus-S-mCherry and S-venus-W-mCherry, venus expression was also lower in the S-venus-W-mCherry strain (abour 83% of the S-venus-S-mCherry), while its RBS remains the same. It shows that the translation of a gene can be influenced by the translation of the adjacent gene on the same operon.

3.3 Tune Translation by Orthogonal Ribosome on Two-gene Operon

To further investigate the influence of the translation of upstream to the downstream gene, we employed a fine tuning method, the orthogonal ribosome method, which was developed earlier in this lab [26]. In short, orthogonal ribosome is a mutated version of the original ribosome, where the rRNA region responsible for recognizing the ribosome binding site was mutated. The orthogonal rRNA was expressed in another plasmid in the host bacterial. It can recruit the ribosomal proteins as wild type and forms the orthogonal ribosome. The orthogonal ribosome only translates genes with the orthogonal ribosome binding site, but not the native ones. And the native ribosomes do not interfere with the orthogonal genes.

The method to tune the translation of a certain gene is to place the orthogonal RBS in front of this gene and control the expression of the orthogonal rRNA expression by aTc inducible promoter. In this way, the gene expression is controlled according to the inducer aTc we add. The plasmid pZE-O-venus-S-mCherry contains venus under the orthogonal RBS and mCherry

under a strong native RBS. This plasmid together with pOR1, which contains orthogonal rRNA under aTC inducible promoter, was co-transformed into *E.coli*. And their fluorescence were measured (Fig. 2).Venus was controlled by aTc concentration as expected. The downstream gene mCherry was coupled to the translation of its upstream gene venus, although mCherry gene and its own RBS were the same. In this construct, mCherry was 55 bp away from end of venus, and venus was equipped with double stop codon. What is more, the orthogonal ribosome, by its nature does not favor the translation of gene with native RBS. Therefore the mCherry translation is not the result of orthogonal ribosome's read through from its upstream venus. This experiment shows that the downstream gene is tightly coupled to its upstream even it is well separated from the upstream genes.

3.4 The mRNA of Two-gene Operon

The mechanism of why downstream translation is conditional to the upstream is to be further investigated here. Our hypothesis is that the ribosomes translating upstream gene protect mRNA from nuclease degradation. Here the mRNA levels of S/W-venus-S-mCherry series are shown in Fig. 3. It shows that when venus is being translated heavily, its mRNA abundance is much more than the construct where venus is weakly translated. Both mRNA measurements on venus or mCherry confirm the above conclusion. The absolute mRNA abundance value between venus and mCherry mRNA have variation, because of different primers used for RT-PCR and experimental error.

The mRNA level of O-venus-S-mCherry strain was also analyzed (Fig. 4). Each sample was extracted for total RNA right after fluorescence measurements. The mRNA level again correlated with the fluorescence of both proteins. All data were normalized to the value from the

maximum induction culture samples. And the mRNA was measured according to mCherry's mRNA. Venus mRNA also shows the same trend. It confirms again that the more ribosome translating the upstream gene, the stable the mRNA gets, therefore the more mRNA there exists in the cell.

3.5 Tables

Table 4. Venus expression with different ribosome binding site

Description	Fluorescence/OD600 (A.U.)
pPROTet-S-venus	35436 ± 2826
pPROTet-M-venus	333 ± 44
pPROTet-W-venus	159 ± 9

3.6 Figures

Figure 1. Construction of plasmid a) and fluorescence and optical density measurements of these strains b). Blue regions in a) represent ribosome binding sites, either strong or weak.

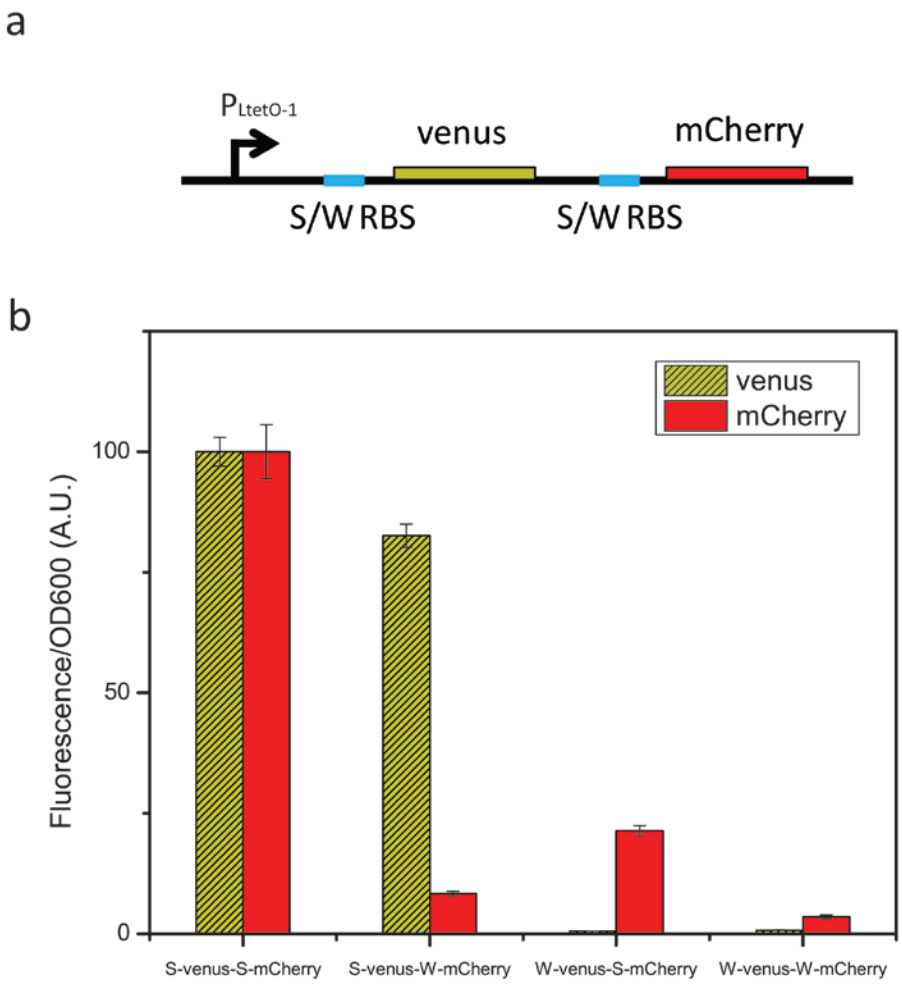


Figure 2. Fluorescence and optical density measurements of O-venus-S-mCherry. The inducer aTc induced the expression of orthogonal ribosome, in turn controlled the expression of venus.

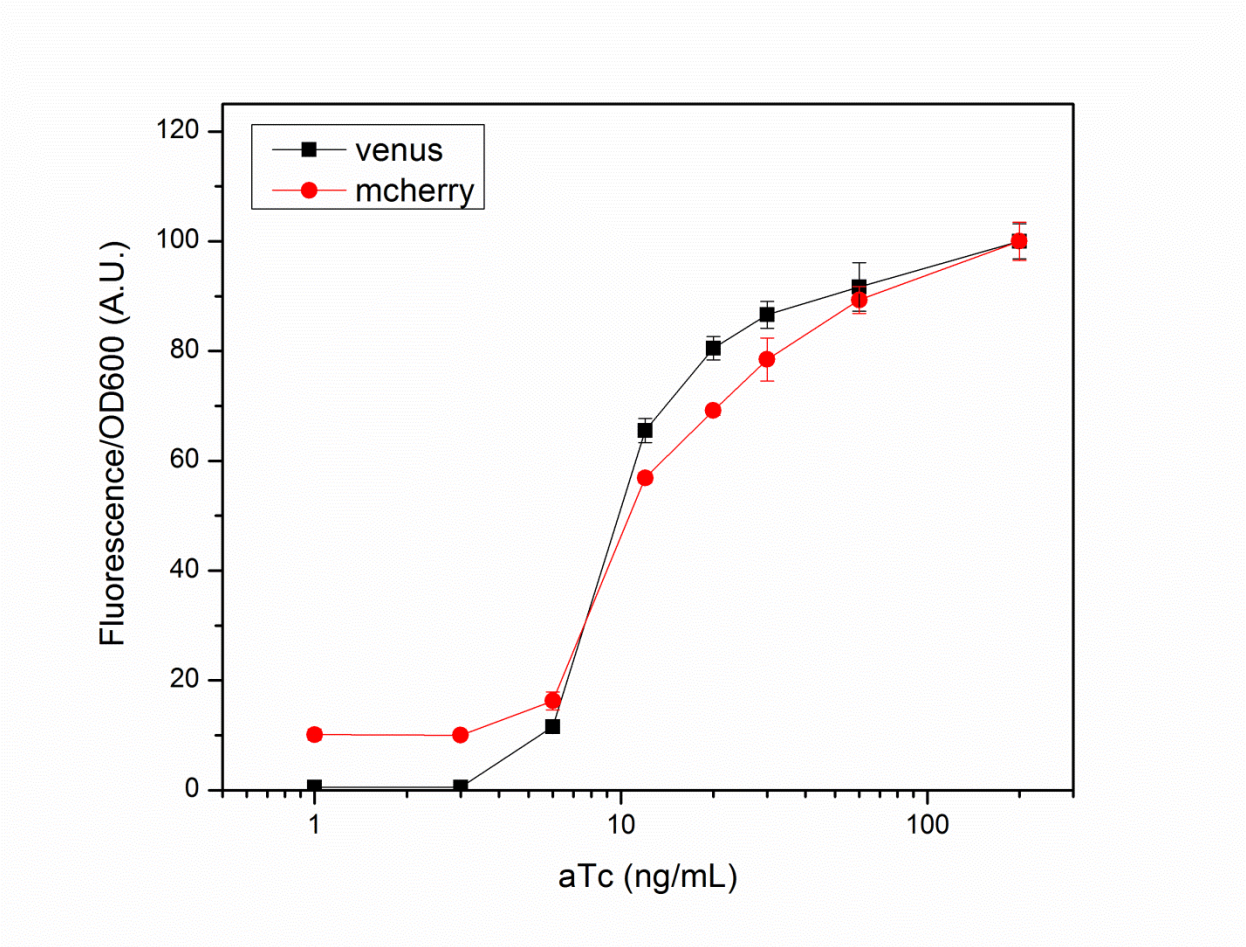


Figure 3. mRNA abundance of the S-venus-S-mCherry and W-venus-S-mCherry strains. The mRNA are measured according to venus sequence (upper panel), and according to mCherry sequence (lower pannel).

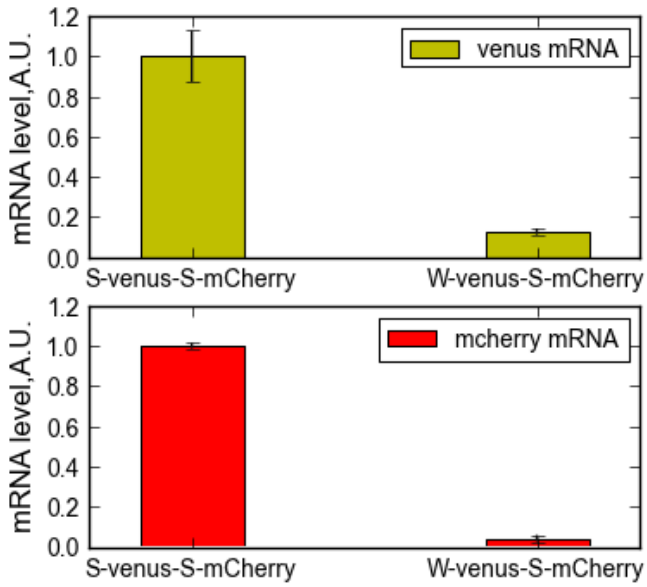
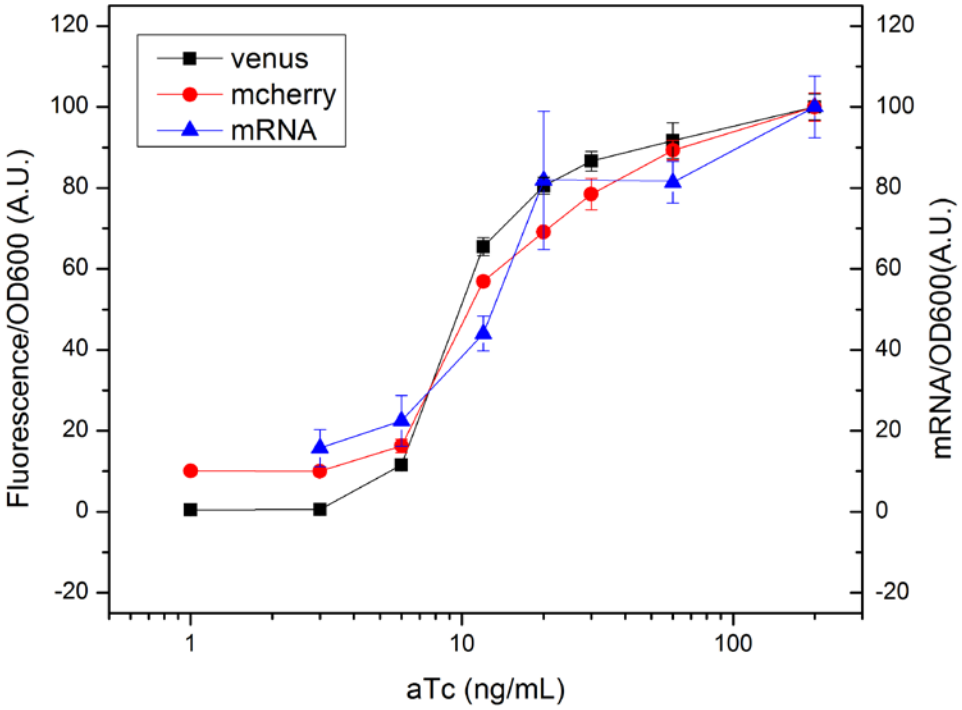


Figure 4. mRNA abundance merged into the fluorescence of venus and mCherry. Performed in the O-venus-S-mCherry strain.



Chapter 4: Discussion and Conclusion

4.1 Summary of Results

I have shown that on the same mRNA, the translation of adjacent genes can influence each other. And the case where the downstream is conditional to the upstream is further investigated by tuning the upstream gene by orthogonal ribosomes. The protein level, as well as the mRNA level is tightly coupled to the translation strength of the upstream gene. It shows that the ribosome is playing an important role is protecting its mRNA, thus further protect the whole mRNA including adjacent genes sharing the same transcript.

4.2 Discussion of Results

Translational coupling has been identified several decades ago [8, 13]. There are three possible mechanisms postulated to explain this phenomenon. One is mRNA stability. As have shown in this study, the translating ribosome binds mRNA and prevents the RNase from degrading it, thus leads to the increase of the mRNA abundance. In polycistronic transcript, the mechanism explains for both upstream protecting downstream and vice verce. But the upstream's influence to downstream can potentially be greater than the downstream's influence to upstream, because in prokaryotes mRNA is transcribed from 5' to 3' direction and the mRNA is being translated right after transcribed. The protection from ribosome right from the very beginning is potentially important to secure the whole transcript. The second mechanism is reinitiation, where the same ribosome translates both upstream and downstream genes. This is typical in the case where the downstream start codon overlaps with the upstream stop codon, such as "ATGA" or "TAATG" [32, 33]. The ribosome is thought to stop at TGA or TAA for the upstream gene and shift back one base / forward two bases and reinitiates at ATG for the downstream gene. Even if the operon

is not in this structure and the ribosome is dissembled from mRNA at upstream stop codon, the local ribosome concentration is still high. This will also enhance the downstream initiation speed potentially. The third mechanism is remodeling. The mRNA is single stranded, therefore tends to fold into secondary structures by complementary bases. The moving ribosome on mRNA of the upstream gene can open the secondary structures, resulting in the exposure of ribosome binding site of the downstream gene. Finally on the other hand, the terminating ribosome may also prevent other ribosome from binding to the RBS of the downstream gene simply by steric mechanism, if the RBS resides too close to the termination site of the upstream gene.

Besides the mechanism of regulation in translational coupling, another interesting issue here is why bacterial evolves to have multiple genes transcibed in one mRNA. In contrast, eukaryotic cell usually organize one gene on one mRNA. Bacterial often have functionally related genes transcribed on one operon. It not only coordinates the regulation of genes, but also helps to locate related proteins in close proximity, resulting in coordinated spatially regulation. As we know bacterial do not have the detailed subcellular division as eukaryotes. The operon structure can provides good spatial regulation in compensation. On the other hand there are also functionally not related genes in the same operon [34]. This is still a mystery left for future study. One explanation is that their protein ancestors might work coordinately but as generations to generations, their functions have evolved into different routes.

This understanding of translational coupling has provided new insights into building synthetic gene circuits. The current design of synthetic gene circuits often relies on transcription regulation, such as promoter engineering. Our finding that the downstream gene is tightly conditional to the upstream gene provides a new way to build linked protein expression. This is done by translation

regulation, thus orthogonal to transcription regulation. It means that we have one more freedom to regulate gene expression. Since more and more tuning methods for translation are bursting, such as siRNA, orthogonal ribosome and codon optimizing and etc, the concept of translational coupling will be more and more useful in terms of gene circuit design.

4.3 Conclusions

Our study provides a detailed analysis of translational coupling in *E.coli*. We constructed a two-gene expression system, where venus and mCherry were under different ribosome binding sites. Furthermore the translation of venus can be tuned by orthogonal ribosomes to achieve controlled expression. In this way we characterized translational coupling in this operon, where the translation of mCherry was tightly conditional to its upstream venus gene. We hypothesized that the ribosome translating the upstream protects the mRNA from degradation, which partially explained why this tight coupling effect. And we have shown the mRNA indeed correlates with the translation of the upstream gene.

Understanding the expression pattern on operons can facilitate engineering non-native gene circuits into bacterial and developing novel biochemical functions.

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